Abstract. Regulation of cytoplasmic p21\(^{\text{CIP1/WAF1}}\) (p21) is of great clinical significance in molecular oncology due to its identification as an antiapoptotic factor, a poor survival predictor as well as a drug-resistance inducer. A retrospective study of the immunohistochemical (IHC) profiles of 128 human primary breast cancers showed that increased total and cytoplasmic p21 expression were highly associated with the expression of I\(^{\kappa}B\) kinase ß (IKKß), the major catalytic subunit of the IKK complex and another crucial player in tumorigenesis and drug resistance. The causal relationship study based on cultured cell lines, MDA-MB-453 and MCF-7, confirmed that IKKß overexpression did upregulate protein levels of total and cytoplasmic p21. Mechanistic investigation demonstrated that IKKß increased p21 expression through upregulation of p21 mRNA level. Moreover, by Western blotting, IKKß was found to be able to upregulate Akt phosphorylation on Ser 473. This novel finding indicated that IKKß could mediate cytoplasmic p21 accumulation via activation of its downstream target Akt, which was known to phosphorylate p21 and lead to cytoplasmic localization of p21.

Introduction

Alternatively named as cyclin-dependent kinase inhibitor-1A (CDKN1A), CDK-interacting protein 1 (CIP1) and wild-type p53-activated fragment 1 (WAF1), p21 is a 21-kDa protein possessing duality in the regulation of cancer behavior. On the one hand, the nuclear form of p21 mainly executes its tumor suppressing function by binding to CDK complexes, inhibiting the activity of cyclin-dependent kinases, such as Cdk2, Cdk3, Cdk4, and Cdk6, and then leading to cell growth arrest (1). In addition, nuclear p21 interacts with proliferating cell nuclear antigen (PCNA) and inhibits DNA synthesis (2). On the other hand, the cytoplasmic form of p21 forms complexes with and inactivates proapoptotic proteins such as the apoptosis signal-regulating kinase 1 (ASK1) (3) and caspase-3 (4-6). Furthermore, cytoplasmic p21 mediates cytoskeletal organization and cell migration through binding and inhibiting the Rho kinase ROCK-1, the malfunction of which leads to loss of the actin stress fibers (7,8). The oncogenic function of cytoplasmic p21 has also been validated by clinical evidence. Higher expression of cytoplasmic p21 has been verified as a factor of poor prognosis in human primary breast carcinomas (9-11). Moreover, resistance to chemotherapeutic drugs may result from accumulation of cytoplasmic p21, as paclitaxel could increase cytoplasmic p21, and reverse the cell cycle inhibitory effect of p21 (12). Thus, cellular localization plays a critical role to determine the function of p21, i.e. nuclear p21, as a cell cycle inhibitor, represses cell growth; cytoplasmic p21, as an anti-apoptotic factor, enhances cell survival. Thus, it is of great interest and importance to understand the regulation of p21.

Cloned independently by several groups early in 1997 (13-15), IKKß was recognized as one of the two catalytic subunits of the IKK complex, which also comprises IKK\(\alpha\), the other catalytic subunit, and the regulatory subunit IKK\(\gamma\)/NEMO. Activated by cytokines such as tumor necrosis factor (TNF)-\(\alpha\) and IL-1, the IKK complex, mainly IKKß (16,17), phosphorylates and triggers I\(^{\kappa}B\) proteins to ubiquitination-dependent degradation, which consequently releases the nuclear factor kappa B (NF-xB) from I\(^{\kappa}B\) in the cytoplasm and activates this transcriptional factor by enabling its nuclear translocation. NF-xB is a well-known transactivator of many antiapoptotic, tumorigenic or angiogenic genes, for instance, cellular inhibitors of apoptosis (cIAPs), cyclin D1 and vascular endothelial growth factor (VEGF). As a result, through activating NF-xB, IKKß plays an important role in promoting...
cell survival, tumor progression and even the development of drug resistance in cancer cells. Additionally, it has been found recently that IKKβ can phosphorylate Foxo3A and inhibit the tumor suppressing function of the latter by inducing nuclear exclusion of this transcriptional factor and causing its degradation through a proteasome pathway (18). As a matter of fact, constitutive activation of IKK has been reported in various human primary cancers and cancer cell lines, including pancreatic cancers, hematopoietic malignancies, and breast cancers (19-21). With all these concerns, more and more effort has been put into developing drugs aimed at IKK-NF-κB pathway, the inhibition of which may have great potentiality in cancer therapy.

In an attempt to further characterize the function of IKKβ in cancer development and progression, we investigated the association between IKKβ and other important molecules involved in cell proliferation, cell cycle regulation, and anti-apoptotic process by IHC studies of human breast carcinoma specimens. Interestingly, the results showed that the expression of IKKβ was correlated with p21 expression, and was correlated with cytoplasmic expression of p21. Our present findings have confirmed for the first time that in primary human breast cancer tissues IKKβ overexpression is associated with increased total and cytoplasmic p21 level. In addition, studies of human breast cancer cell lines provided a plausible explanation for the phenomenon and might help to yield new and promising strategies for p21-attenuation therapy focusing on the regulatory effect of IKKβ-NF-κB pathway.

Materials and methods

Patient population and tissue samples. Human primary invasive breast cancer surgical specimens (128) were obtained from the archives of Department of Pathology, Shanghai East Breast Disease Hospital, P.R. China. All patients received mastectomy and axillary lymph node dissection between 1988 and 1994. The surgical specimens were formalin-fixed, paraffin-embedded, hematoxylin and eosin-stained, and histopathologically diagnosed.

Antibodies, chemicals and plasmids. The antibodies used in this study were p21 (c-19) from Santa Cruz Biotechnology Inc. (Santa Cruz, CA); IKKβ (10AG2) from Oncogene Research Products (San Diego, CA); IKKβ antibody, Akt antibody, and Phospho-Akt (Ser473) (193H12) from Cell Signaling Technology, Inc. (Beverly, MA); monoclonal anti-flag M5 and monoclonal anti-α-tubulin (B-5-1-2) from Sigma (St. Louis, MO); and PARP (C2-10) from BD Biosciences (San Jose, CA). Horseradish peroxidase-labeled anti-mouse and anti-rabbit antibodies were obtained from Jackson Immuno-Research Laboratories, Inc. (West Grove, PA). The avidin-biotin-horseradish peroxidase complex was from Vector Laboratories (Burlingame, CA). 5-μm tissue sections were deparaffinized, rehydrated, and subjected to heat-induced epitope retrieval in 0.01 mol/l citrate buffer (pH 6.0). After digestion in 0.05% trypsin, the sections were incubated in 0.3% hydrogen peroxide for 5 min to reduce the endogenous peroxidase activity and then in 10% normal horse-serum for 30 min at room temperature to block non-specific binding. Next the sections were incubated with the primary antibodies against IKKβ or p21 overnight at 4˚C in a humid box. Following sequential incubation with the biotinylated second antibodies and the avidin-biotin-horseradish peroxidase complex, immunodetection was carried out using the 0.125% 3-amin-9-ethylcarbazole (AEC) substrate solution (Sigma). The sections were then counter-stained with Mayer’s hematoxylin (Sigma), and mounted for microscopic examination. According to the percentage of positively stained tumor cells, IKKβ immunoreactivity was scored from 0 to 2 as follows: 0, <20% cells stained; 1+, 20-49% cells stained; 2+, >50% cells stained. Total p21, as well as cytoplasmic and nuclear p21 staining were classified into two groups based on the percentage of positively stained tumor cells respectively. Low expression of p21 is defined as positive staining in <10% of the tumor cells; and high expression refers to positive staining in greater than or equal to 10% of the tumor cells. Correlations between IKKβ and p21 expression, including the total, cytoplasmic and nuclear forms, were examined by the Pearson correlation test using SPSS 10.0 software. All P-values were two-sided and P<0.05 was set as the cutoff for statistical significance.

Cell lines, cell culture and transfection. MDA-MB-453 and MCF-7 cell lines were obtained from the American Type Culture Collection (Manassas, VA). Electroporation-based transfection was carried out in MDA-MB-453 cell lines using Nucleofector I (Amaxa, Cologne, Germany) according to the manufacturer’s instructions. SN, a cationic liposome reagent, was used in the transfection for MCF-7 and MEF cell lines. MDA-MB-453 and MCF-7 cell lines stably transfected with pCDNA3 (MDA-MB-453-v1 and MCF-7-v2) or IKKβ (MDA-MB-453-B16, B20, and MCF-7-B5, B12), were established as described previously (23). All cell lines were maintained in Dulbecco’s modified Eagle’s medium containing high glucose (DMEM/F-12) with 10% fetal bovine serum, except the stable cell lines were grown in additional 0.006 mg/ml blasticidin (Invitrogen, Carlsbad, CA).

Western blotting and cell fractionation. For Western blot analysis, the cells were lysed in lysis buffer containing 2X RIPA-B buffer (2% Triton X-100, 300 mM NaCl, 40 mM Na2PO4, pH 7.4), with 100 mM NaF, 2 mM Na3VO4, 1 mM phenylmethylsulphonyl fluoride (PMSF), and 0.075 TlU/ml aprotinin. Then the total lysates were separated by SDS/PAGE and transferred to nitrocellulose membranes. Following
blocking in 5% non-fat dry milk or 5% BSA in 0.05% Tween PBS, the membranes were sequentially incubated with primary antibodies and horseradish peroxidase-conjugated secondary antibodies. Finally the immunoblots were visualized using an enhanced chemiluminescence kit (Amersham Pharmacia Biotech).

Cell fractionation was carried out as follows: Cells were harvested in cell lysis buffer (20 mM HEPES, pH 7.9, 10 mM KCl, 1.5 mM MgCl₂, 0.5 mM DTT, 0.5 mM PMSF, 100 mM NaF, 2 mM Na₃VO₄, 0.075 TIU/ml aprotinin). Then the cells were homogenized in a 10 ml Dounce homogenizer on ice. Microscopic evaluation was used to ensure the purity of the nuclei and cytoplasm. Following centrifugation at 1,200 g for 5 min, the supernatant was again centrifuged at 16,100 g for 10 min at 4ºC, and the resulting supernatant was collected as the cytoplasmic fraction. The pelleted nuclei collected after the first centrifugation were washed five times with the washing buffer (20 mM HEPES pH 7.9, 10 mM KCl, 20 mM MgCl₂, 0.5% NP40) and then resuspended in the nuclear lysis buffer (250 mM NaCl, 0.1% NP40, 50 mM HEPES pH 7.9, 0.5 mM DTT, 1 mM PMSF, and 0.075 TIU/ml aprotinin). The nuclear lysates were collected following sonication and centrifugation at 16,100 g for 10 min at 4ºC. Both the cytoplasmic and nuclear fractions were subjected to Western blotting. The fractionation efficiency was assessed by antibodies against α-tubulin and PARP.

**Luciferase reporter assay.** WWP-Luc, the firefly luciferase reporter under the control of the p21 promoter, was transfected into the MDA-MB-453 stable cell lines, as well as the wild-type and IKKβ knockout MEF cell lines. Renilla luciferase reporter pRL-TK (Promega) was used as the internal control. Forty-eight hours after transfection, cells were harvested and subjected to luciferase reporter assay using the dual luciferase assay kit and the TD20/20 luminometer (Promega) according to the manufacturer’s instructions.

**RT-PCR.** Total RNAs of MDA-MB-453 and MCF-7 stable cells were extracted by TRIzol reagent (Invitrogen) according to the manufacturer’s instructions. First-strand cDNA was generated using oligo-(dT) and SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen). The resulting cDNA was then subjected to PCR using following conditions: pre-denaturing at 95ºC for 2 min and 20-24 cycles of 95ºC for 10 sec, 55ºC for 10 sec, and 72ºC for 10 sec. The primers for p21 and the internal control GAPDH gene were 5'-CAGGGACAGCAGGGAAGA-3' and 5'-GGACGCTCTCTTGAGAAA-3' (24); 5'-AACCATGAGAAGTATGACAAC-3' and 5'-GTCATACGAGAATGACTC-3' (25), respectively. The PCR products were resolved in 2% agarose/ethidium bromide gels.

**Results**

IKKβ overexpression is associated with both increased total and cytoplasmic p21 expression in human breast cancer tissues. A retrospective study was carried out using the existing IHC profiles of 128 primary breast cancer samples. IKKβ and p21

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<tr>
<th>IKKβ</th>
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<th>2 (%)</th>
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<td>Total p21</td>
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<td>Low</td>
<td>44 (60.3)</td>
<td>9 (50.0)</td>
<td>12 (32.4)</td>
<td>65 (50.8)</td>
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<td>High</td>
<td>29 (39.7)</td>
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<td>25 (67.6)</td>
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<td>P=0.006*</td>
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| Cytoplasmic p21 |       |   |       |   |       |   |           |   |
| Low | 52 (71.2) | 12 (66.7) | 14 (37.8) | 78 (60.9) |
| High | 21 (28.8) | 6 (33.3) | 23 (62.2) | 50 (39.1) |
| P=0.001* | | | | |

| Nuclear p21 |       |   |       |   |       |   |           |   |
| Low | 52 (71.2) | 11 (61.1) | 23 (62.2) | 86 (67.2) |
| High | 21 (28.8) | 7 (38.9) | 14 (37.8) | 42 (32.8) |
| P=0.309* | | | | |
| Total | 73 (100.0) | 18 (100.0) | 37 (100.0) | 128 (100.0) |

*Pearson’s correlation test.
immunoreactivity of the same patient samples were previously examined and published separately for other purposes (10,18). To better determine the possible relationships between IKKβ and p21, we graded the immunoreactivity of IKKβ and p21 according to the percentage of positively stained tumor cells as described in Materials and methods, and evaluated their association by the Pearson's correlation test. Statistically IKKβ level was associated with total p21 expression (P=0.006, Table I and Fig. 1A). As the role of p21 in cancer development greatly depends on its subcellular localization, we also examined whether IKKβ might affect cytoplasmic and/or nuclear p21 expression. The finding was that IKKβ level was correlated with cytoplasmic p21 expression (P=0.001, Table I and Fig. 1B), but not with nuclear p21 expression (P=0.309, Table I and Fig. 1C). Representative cases showing the relationship between IKKβ and p21 expression are shown in Fig. 1D.

IKKβ overexpression elevates total and cytoplasmic p21 levels in cell lines. Since the IHC data demonstrated correlations between IKKβ overexpression and p21 expression both in the total and cytoplasmic forms, we then examined whether IKKβ could cause p21 expression change by studies of cell lines. First, the protein levels of IKKβ and p21 were analyzed by Western blotting using total cell lysates of breast cancer cell lines MCF-7 and MDA-MB-453, stably transfected with pCDNA3 vectors (MCF-7-v2/MDA-MB-453-v1) or flag-tagged IKKβ (MCF-7-ß5 and 812/MDA-MB-453-ß16 and ß20). p21 level was obviously higher in the IKKβ transfectants than in the pCDNA3 transfectants (Fig. 2A). Additionally p21 expression dramatically decreased in IKKβ knockout MEF cell lines than in the wild-types (Fig. 2B). Furthermore, cellular fractionation and Western blot analysis were carried out using the stable cell lines to determine p21 protein levels in different locations. Comparing the IKKβ transfectants and the vector controls, p21 expression was significantly increased in the cytoplasmic fractions but not in the nuclear fractions (Fig. 2C).

IKKβ overexpression increases p21 mRNA level and transactivates p21. To further investigate how IKKβ functions in the regulation of p21 expression, we first examined whether p21 protein turnover rate was disturbed by IKKβ. Both MDA-MB-453 and MCF-7 (data not shown) stable cell lines were treated with cycloheximide (Sigma) for 0, 1, 2, 3, 4, and 5 h. Cell lysates were then harvested and blotted with the anti-p21 antibody. Quantitation of the immunoblots showed that p21 half-lives of the IKKβ, stable cell lines and that of the vector controls had no obvious difference (Fig. 3A). We next examined whether p21 expression may be regulated
Figure 2. IKKβ overexpression elevates total and cytoplasmic p21 levels in breast cancer cell lines. A, MDA-MB-453 and MCF-7 cell lines were stably transfected with pCDNA3 vectors (MDA-MB-453-v1 and MCF-7-v2) or flag-tagged IKKβ (MDA-MB-453-ß16, ß20, and MCF-7-ß5, ß12). p21 protein levels were obviously higher in the IKKβ transfectants than in the pCDNA3 transfectants. B, p21 expression dramatically decreased in IKKβ knockout MEF cell lines than in the wild-types. C, p21 levels in both cytoplasmic and nuclear fractions of the MDA-MB-453 stable cell lines were determined by cell fractionation and the following Western blot analysis. Comparing the IKKβ transfectants and the vector controls, p21 expression was significantly increased in the cytoplasmic but not in the nuclear fractions. Band density was quantitated and p21 expression was normalized to loading controls, such as cytoplasmic expression of α-tubulin and nuclear expression of PARP.

Figure 3. IKKβ overexpression increases p21 mRNA level and transactivates p21. A, IKKβ overexpression did not affect the p21 protein turnover rates. After treatment with cycloheximide (10 μg/ml, Sigma) for 0, 1, 2, 3, 4, and 5 h, MDA-MB-453 stable cell lines were harvested for Western blot analysis. Normalized to the loading controls, the quantitated data showed no obvious difference between the p21 half-life of the IKKβ transfectant and that of the vector transfectants. B, IKKβ overexpression increases p21 mRNA level. RT-PCR was used to compare the mRNA levels in both MCF-7 and MDA-MB-453 stable cell lines. Band density was quantitated and p21 expression was normalized to loading controls. Higher p21 mRNA expression was detected in the IKKβ than in the vector transfectant. C, p21 was activated by IKKβ at transcriptional level. The WWP-Luc plasmid, a luciferase reporter gene under the control of p21 promoter, was transfected into the wild-type and IKKβ knockout cell lines using SN, a cationic liposome reagent. The Renilla luciferase reporter pRL-TK was used as the internal control. Forty-eight hours after transfection, luciferase reporter assay was performed using the dual luciferase assay kit.
at the mRNA level. To this end, RT-PCR was used to compare the mRNA levels in the IKKβ stable cell lines and the vector controls. Higher mRNA expression was detected in the IKKβ than in the vector transfectants of both MCF-7 and MDA-MB-453 cell lines (Fig. 3B). To further determine whether IKKβ is involved in transcriptional activation of p21, the WWP-Luc plasmid, consisting of a 2.4-kb wild-type p21 promoter and a fused luciferase reporter gene, was transfected into the MDA-MB-453 stable cell lines. The relative luciferase activity was higher in the IKKβ stable cell lines than in the vector control (Fig. 3C). The same experiment was also done using the wild-type and IKKβ knockout MEF cell lines. The relative luciferase activity was lowered in the IKKβ-/- cells, suggesting that p21 was transcriptionally activated by IKKβ (Fig. 3C).

IKKβ overexpression increases cytoplasmic p21 expression via upregulation of phosphorylated Akt. Next we asked how IKKβ influenced cytoplasmic p21 expression. It has been shown previously that p21 can be phosphorylated by Akt and results in its cytoplasmic localization (26). Phosphorylated Akt, which is the active form, promotes cell growth, proliferation and survival through its own serine/threonine kinase activity. To test whether IKKβ may upregulate Akt phosphorylation thus leading to accumulation of cytoplasmic p21, we examined total Akt and phosphorylated Akt (Ser 473) levels in MDA-MB-453 stable cell lines using Western blot analysis. There was virtually no change of the total Akt levels in all samples. However, phospho-Akt expression was significantly higher in the IKKβ stable transfectants than in the vector controls (Fig. 4A). Increased Akt phosphorylation was also observed in MCF-7 IKKβ stable cell lines (data not shown). To further examine whether IKKβ activity is required for Akt activation, we treated the cells with the IKK inhibitor Wedelolactone (100 μM, 2 h at 37˚C), and indeed detected reduced Akt phosphorylation (Fig. 4B), reinforcing the notion that IKKβ could increase Akt activity. Next, to see whether IKKβ-upregulated cytoplasmic p21 expression could be reduced via inhibition of Akt activity, the MDA-MB-453 stable cell lines were treated with or without the specific PI-3 kinase inhibitor LY294002 (40 μg/ml, 4 h at 37˚C), and then harvested for cell fractionation and Western blotting. The results showed more dramatic downregulation of cytoplasmic p21 in the IKKβ stable transfectants than that in the vector control (Fig. 4C), thus supporting that Akt activation is involved in IKKβ-mediated cytoplasmic p21 accumulation.

Discussion

Our results show that IKKβ increases the p21 protein level, and this upregulation is caused by elevation of the p21 mRNA level and transcriptional activation of p21. Several studies have shown that p21 expression is increased by NF-κB activation in different kinds of cell lines, including monocytes, Ewing tumor cells, HeLa cells as well as normal human epithelial cells (27-29). In addition, an NF-κB binding site was found in the human p21 promoter (30). Therefore IKKβ may transcriptionally upregulate p21 through the activation of
NF-κB, which is one of the most important IKKß downstream targets.

What is more clinically relevant and significant of this study lies in the finding of a novel regulation mode of the cytoplasmic p21 expression in human cancers, i.e. via IKKß-mediation. Indeed, we found that IKKß could upregulate Akt phosphorylation on Ser 473, and then the activated Akt phosphorylates p21 on Thr 145 within its nuclear localization signal (NLS) and blocks the translocation of p21 into the nucleus, leading to p21 cytoplasmic accumulation. In cases other than cancers, previous studies of monocyte differentiation showed that NF-κB activation accompanied by increased IKKß kinase activity in differentiated macrophages protects the cells from differentiation-induced apoptosis through upregulation of p21, which was located in the cytosol of the macrophages and having anti-apoptotic function (3,28). However, it remains unclear whether and how IKKß-NF-κB pathway is able to cause p21 accumulation in the cytoplasm, rather than in the nucleus of the differentiated macrophage. Now our study mainly based on human cancer tissues and cell lines may provide a plausible explanation for these phenomena, i.e. when monocytes differentiate into macrophages, the accompanying IKKß activation may cause Akt phosphorylation and the consequent accumulation of the ‘prosurvival’ p21 in the cytoplasm. Despite a report showing that no activated Akt detected in the monocyte differentiation system stimulated by vitamin D3 (31), supporting evidence came from the findings that Akt was constitutively active in human macrophages and played an important role in protecting the macrophages from apoptosis (32). In addition, inhibition of the PI-3K/Akt pathway was shown to induce apoptosis of murine peritoneal macrophages (33).

Collectively, our study demonstrated the involvement of IKKß in the regulation of both total and cytoplasmic p21 expression. We showed that IKKß could enhance p21 mRNA level and transcriptional activation. Although further exploration is required to clarify the underlying mechanism, we found for the first time that IKKß upregulated Akt phosphorylation on Ser 473. This novel finding provided the notion that IKKß-mediated cytoplasmic p21 regulation may involve the activation of Akt. In view of the facts that p21 may become a promising target for anti-cancer treatment, and current approaches toward p21 ablation are quite limited, mainly focusing on utilizations of antisense p21 oligodeoxynucleotide (ODN) or plasmids encoded by antisense p21 to decrease p21 expression (34). Therefore our study provides the feasibility of making p21 another target of IKKß-NF-κB inhibition therapy, which can be achieved through diversified methods, including the use of IKKß or NF-κB-oriented specific small molecule inhibitors which are more therapeutically applicable than the antisense techniques currently dominating in the p21 attenuation studies.

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References


